The Living Scar – Cardiac Fibroblasts and the Injured Heart

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Abbreviations: AP – action potential**, BM** – bone marrow, **Cav** – caveolin **, Cx** – connexin, **ECM** – extracellular matrix, **EMT** – epithelial-to-mesenchymal transition, **EndMT** – endothelial-to-mesenchymal transition, **FGF** – fibroblast growth factor, **GFP** – green fluorescent protein, **IL** – interleukin, **LOX** – lysyl oxidase, **MCP-1** – monocyte chemoattractant protein, **MI** – myocardial infarction, **TGF-β** – transforming growth factor-β

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This review explores available insight and recent concepts on fibroblast integration in the heart, and highlights potential avenues for harnessing their roles to optimise scar function following heart injury such as infarction, and therapeutic interventions such as ablation.

1. The Scar – a Living Tissue

1.1 Scar Formation

When considering cardiac structure and function, the focus is usually on muscle cells, even though non-myocytes form the majority of cells in the heart. Non-myocytes include multiple cell types, the largest of which are endothelial cells and fibroblasts [1]. Fibroblasts are a heterogeneous and dynamic group of cells which are known to be important for developmental, structural, and biochemical integrity of the heart, as well as for tissue-repair and/or reactive processes as observed in scar formation and genetic hypertrophic cardiomyopathies, respectively (for reviews see [2-6]). In spite of this, fibroblasts have often been seen as less interesting than their cardiomyocyte cousins.

Although myocardial infarction (MI) may be the most common cause of ventricular scarring in humans, scars also occur in non-ischaemic cardiomyopathies due to replacement **fibrosis** (see "Glossary") during both **pressure/volume overload** [7] and normal ageing [8], although aging is not necessarily associated with fibrosis *per se* [9]. In addition, scars result from clinical interventions such as ablation and surgical procedures [10] (see Box 1).

Of note, the discussion about scars and fibrosis is confounded by the fact that these terms are often used interchangeably. 'Fibrosis' is *not* synonymous with elevated presence of interstitial cells: it is quantified through presence of collagen – a key component of the *acellular* fraction of connective tissue (Fig. 1A).

Fibrotic scars, such as in skin, are generally acellular and predominantly composed of fibrillar collagen [11]. In the heart, however, scar tissue assumes a more pro-active role than simply preserving ventricular integrity, facilitating force transmission, and preventing rupture. Nonetheless, myocardial scarring does share common mechanisms and morphological milestones with classic wound healing (reviewed in [4, 12]). Briefly, injury is followed by spreading tissue necrosis, neutrophil infiltration, and macrophage-driven clean-up of cellular debris. Subsequently, granular tissue formation, neovascularisation, and (partial) sympathetic re-innervation occur. Infiltration (from intra- and extra-cardiac sources; see section 2.3) and proliferation of fibroblast-like cells occurs throughout, and is observed as early as a few hours post-injury [13, 14]. Large amounts of newly produced collagen act to reinforce the healing tissue, eventually establishing a steady state involving balanced extracellular

matrix (ECM) production by fibroblasts and degradation *via* matrix metalloproteinases that are released by leukocytes, fibroblasts, and smooth muscle cells [15]. The traditional view of scar formation (based on observations in organs such as skin) suggests that healing is followed by apoptosis of the vast majority, if not all, of the cells (including fibroblasts), leaving a mature, fibrillar scar. This whole process takes several weeks post-injury, and – in the heart at least – takes place in an environment of rhythmically changing stress and strain.

1.2 The Living Scar

Despite prevailing perceptions, cardiac scars are dynamic living structures [16, 17]. The abundantly present ECM is interlaced with phenotypically diverse groups of cells: interstitial fibroblast-like cells (both functionally and structurally heterogeneous, see section 2), endothelial cells, vascular smooth muscle, surviving cardiomyocytes, immune cells, neurons, and adipocytes [18, 19] (Fig. 1B,C). The scar is a metabolically dynamic tissue which furthermore exhibits non-linear passive and active mechanical properties (of course, 'active' force-generation by non-myocytes occurs over time at scales that are orders of magnitude longer than the heartbeat) [20]. Contractile properties of the scar rely on the presence of non-vascular, α-smooth muscle actin-expressing non-myocytes, which persist in cardiac scars for many years following injury such as MI [21-23] (note: not all subsets of fibroblasts express contractile proteins [24]), as well as on the presence of an extensive cytoplasmic fibrillar system of cell-to-cell and cell-to-ECM attachments [25].

The impact of scar tissue on cardiac electrical activity is a matter of debate [26]. Fibrosis can exhibit variable degrees of density, from focal and compact (in the case of scars) to patchy and diffuse (Fig. 1A). This can lead to separation of strands of myocardium, forcing excitation waves to take anisotropic, circuitous paths [27] that may set the stage for **re-entry** of **excitation** [28]. Although fibrosis is strongly associated with elevated risk of arrhythmogenesis, it is not well understood how exactly it is involved in either the active generation or the passive maintenance of abnormal electrical conduction episodes.

Commonly, the effect of connective tissue on cardiac electrophysiology has been attributed to its non-excitability and resulting electrical insulation. Without question, fibrosis can create areas of conduction block and define structural anchors of re-entry circuits [29, 30]. However, certain clinical observations suggest that scars are not necessarily always and exclusively arrhythmogenic electrical insulators. Thus, no heart in the aged is likely to be devoid of scars [31], so one may wonder why these appear to not be arrhythmogenic. Perhaps more remarkably, **atrial ablation lines** become electrically transparent over time in a majority of patients [32], suggesting the possibility of trans-scar conduction of electrical excitation. While ablation lines may be structurally incomplete, even if intra-procedurally they appear continuous,

this reservation does not apply to fully-**transmural post-surgery scars**. Even in this setting, trans-scar conduction has been reported in up to 20% of patients, for example across suture lines after transplantation or after repair of cardiac birth-defects [33, 34]. Whatever the substrate of trans-scar coupling, the underlying electrical connections are formed *de-novo* post-surgery.

Approaches to fixing injured myocardium typically have been geared towards re-muscularisation, whether through transplantation of stem/progenitor-derived cells into scarred areas, attempts to induce endogenous neomyogenesis *via* division of existing myocytes, or trans-differentiation of non-myocytes (including fibroblasts) into myocytes. Frustratingly, only limited success has been seen with these efforts.

The challenges associated with generating new cardiac muscle raise an obvious alternative: to make better scars.

Before exploring this option, however, it may be instructive to consider the nature, source, and roles of the fibroblasts that populate cardiac scar tissue.

2. Scar Fibroblasts – What Are They and Where Do They Come From?

2.1 Properties of Cardiac Fibroblasts

Fibroblasts play a prominent role in defining cardiac structure and function. They are sources and targets of signalling cascades, including chemical, mechanical, and electrical signals, involving cellular and acellular components of the heart.

Fibroblasts may be defined as *non-excitable cells of mesenchymal origin that produce interstitial collagen*. Morphological identifiers include a lack of basement membrane, and the presence of multiple elongated cytoplasmic processes or sheet-like extensions and irregular folds. These can bring the total surface area of cardiac fibroblasts *in vivo* to 1,500 μm^2 or more [35, 36]. Fibroblasts are arranged within the extracellular space in complex 3D sheaths that surround and enmesh myocytes, as well as vascular structures and other non-muscle cells [5, 37].

It is well established that fibroblasts are phenotypically heterogeneous, and that their cellular characteristics depend on their developmental stage and physiological conditions [38, 39]. For example, the density of fibroblasts and their responsiveness to growth factors differ between atria, ventricles and valves [40]. Unfortunately, this heterogeneity means that no single fibroblast marker presently allows cell-identification that is specific (i.e. marking only fibroblasts) and inclusive (i.e. marking all fibroblasts in the heart). This includes commonly used markers such as discoidin domain collagen receptor, fibroblast-specific protein 1, fibroblast

activation protein, platelet derived growth factor receptor alpha, periostin, Thy1 cell surface antigen, and vimentin (reviewed in [41, 42]).

2.2 Fibroblast Activation

Resident cardiac fibroblasts have little or no contractile microfilaments or stress fibres [43]. Early during scar formation, fibroblasts become activated and undergo phenotype transition into myofibroblasts [3, 44, 45]. They then acquire a migratory phenotype, commence expressing *α*-smooth muscle actin, develop contractile bundles, and exhibit altered **connexin** distribution [13, 46]. However, since fibroblasts are pleiomorphic by nature, there is no defined threshold at which 'a fibroblast becomes a myofibroblast' (increased contractile filament content does not transform a fibroblast into a different cell type, and myofibroblasts do not have unique lineages separate from fibroblasts). For that reason we will be using the general term "fibroblast" in reference to all of its phenotypes across the spectrum throughout this review.

There are several ways to activate fibroblasts, a major trigger being changes in the mechanical and structural microenvironment, for example as a result of a loss of myocardial histological integrity post-injury [47]. It is worth noting that *ex vivo* cultured fibroblasts are generally 'activated'. Another important signal for fibroblast activation is TGF-β signalling [48]. The functional consequences of cardiac fibroblast activation include increased proliferation and migration [49]; increased responsiveness to, and release of, signalling molecules; deposition of ECM; changes in the expression of adhesion molecules (such as integrins) and their receptors [50]; and changes in the expression of other matricellular proteins (for example periostin, osteopontin, tenascin C) [51]. Additionally, fibroblast activation is associated with an increase in mitochondrial content and respiration [52].

2.3 Origins of Activated Cardiac Fibroblasts

We acknowledge that historically fibrosis is perceived as resulting from the cytokine-driven activation of "resident" fibroblasts into myofibroblasts [53] (although residency does not identify origin). A question presently under investigation is whether all fibroblasts in the adult heart are carried over from embryonic life or, if as suggested by recent studies, fibroblasts in the adult heart are additionally derived from cells of bone marrow origin or from epithelial cells including endothelium, pericytes or epicardium [42, 54, 55]. As a result, the contribution of different cell sources in the aftermath of cardiac injury is a matter of debate. Additionally, some studies also highlight the role of fibroblast senescence in fibrotic response to injury [56]. Investigations into the exact make-up of scars have been hindered by the lack of clear-cut lineage studies, needed for sharp delineation of non-myocyte origins.

Subsets of epicardial cells have been shown to activate and transition into cardiac fibroblasts after acute cardiac injury (such as murine infarction) though epithelial-to-mesenchymal transition (EMT) [57-59], as seen also during embryonic development [60, 61]. These adult EMT-derived fibroblasts tend to reside in the sub-epicardial space, expressing collagen and contributing to a pro-fibrotic repair response. Consequently, inhibition of EMT leads to cardiac chamber dilatation and worsening **ejection fraction**, suggesting that epicardially-derived fibroblasts play important roles in cardiac repair, at least in murine ischemic injury model [62, 63]. This relevance may be disease-specific [57].

Infarcted and non-infarcted models of cardiac fibrosis have also suggested a role for endothelial-to-mesenchymal transformation (EndMT) [64]. EndMT has been reported to contribute up to 30% of fibroblasts in a murine model of pressure overload injury [64]. The degree to which EndMT is relevant for repair in the acutely injured heart is less certain, with several studies finding no evidence for an involvement of EndMT in cardiac repair [49, 65].

Additionally, pericytes (epithelial-like cells that envelop endothelial cells in non-muscular microvessels and capillaries) could contribute to the pool of cardiac fibroblasts post-injury [54, 66, 67]. Some studies suggest that around 10% of activated fibroblasts in MI scars are pericyte-derived [21, 68].

Finally, a significant proportion (between a quarter [69] and two thirds [55]) of fibroblasts in post-injury scars appear to be of bone marrow (BM) origin [70, 71]. Involvement of BM-derived cells in cardiac repair has been highlighted by work involving chimeric mice, where the BM of lethally irradiated animals was reconstituted by a single clone of green fluorescent protein positive (GFP^+) hematopoietic stem cells (rigorously isolated from the Okabe EGFP+ transgenic mouse that expresses EGFP in all cells). BM-derived cells could thus be tracked with certainty (by GFP fluorescence), and were found to give rise to *bona-fide* activated fibroblasts, both activated and quiescent, in the heart [71-73]. Pre-homing, these circulating precursors were shown to express hematopoietic (CD45), monocytic (CD11 and CD14) and progenitor markers (CD34), as well as collagen-1 mesenchymal marker [74-76]. In contrast, other studies have suggested that BM contributions to the cardiac fibroblast populations after injury is minor, or marks a transition from reparative fibrosis to malignant scarring in the infarcted heart [42, 49, 57]. One possible explanation to these differences is that $CD45^+$ cells expressing fibroblast markers may downregulate expression of the CD45 surface protein following engraftment [77], or there may be other technical issues. For example, it is unclear from reports using the *Vav*-cre [49] mouse model whether the Cre-driver was able to activate all, or just subsets, of hematopoietic progenitor cells that was seen with CD45-cre:YFP mice [78] (due potentially to tissue-specific splicing mechanisms, differences in epigenetic remodelling during differentiation, or other

factors that affect transcription of recombinase in immature hematopoietic stem cells). Results obtained using the EGFP transgenic mouse in single cell engraftment experiments did not depend on Cre expression or antibody staining to demonstrate the engraftment of bone marrow cells into a non-myocyte population found in the adult heart.

Injury-induced recruitment and activation of fibroblasts from such a diverse pool underlines the importance of these non-myocytes in cardiac self-repair, particularly when considering remedial therapies. Unfortunately, no fully comprehensive study so far reports the exact proportions in the healing myocardium of fibroblasts from the different sources in different injury models.

2.4 Destination of Activated Cardiac Fibroblasts

In addition to the uncertainty about sources, the timing and proportion of various fibroblasts arriving at the site of cardiac injury is a matter of debate. Equally, although migration of fibroblasts into the region of cardiomyocyte loss is crucial for scar formation, the molecular signals directing fibroblast migration remain poorly understood.

We do know that chemokine/chemokine receptor interactions stimulate fibroblast progenitor chemotaxis into the infarct. One candidate chemokine is the monocyte chemoattractant protein (MCP)-1/CCL2. Cardiac overexpression of MCP-1 induces myocardial IL-6 secretion and accumulation of cardiac fibroblasts, thereby preventing the development of cardiac dysfunction and adverse remodelling after murine infarction [79]. In a mouse model of ischemic cardiomyopathy, repetitive ischemia/reperfusion episodes resulted in fibrotic cardiomyopathy concurrent with markedly prolonged induction of MCP-1 and increased presence of small spindle-shaped cells in the myocardium that express collagen I, α-smooth muscle actin, CD34, and CD45. In this setting, left ventricular dysfunction could be prevented by either genetic deletion of MCP-1 or injection of a neutralizing anti-MCP-1 antibody [80, 81].

Growth factors (such as TGF-β and FGF) may also trigger migration of fibroblasts to the site of injury [48]. In addition to pro-migratory pathways, inhibitory signalling factors such as CXC chemokine CXCL10/Interferon-γ-inducible Protein-10 (which curbs fibroblast migration), are also activated in the infarcted myocardium, presumably countering excessive fibrotic responses [82, 83].

Once the activated fibroblasts arrive at the site of injury, they do not simply assume a random position and orientation. In transmural infarctions, for example, activated fibroblasts orientate in planes parallel to endo- and epicardium, whereas in non-transmural patchy scars they show an orientation that follows adjacent

cardiomyocyte directions, suggesting that mechanical cues act on the cells, encouraging them to align in a specific manner [22].

A question equally important to “What makes fibroblasts come?” is “What makes them stay?” In tissues such as skin, scar fibroblasts die off, once the scar is stable and the associated inflammation is resolved. In the heart, however, a significant proportion of cells persist in scar tissue for years after injury [22]. Their persistence in other injured organs is associated with progressive fibrosis and predicts organ failure (for example toxic nephritis [84])**.** However, in the heart the opposite seems to occur: strategies aimed at decreasing fibroblast apoptosis report favourable effects on murine infarct healing, cardiac function post-infarction, and survival [85].

Therefore, manipulation of homing, arrival, activation, and perseverance of scar fibroblasts presents highly enticing, if complex, therapeutic targets.

3. The Many Roles of Scar Fibroblasts

Fibroblasts contribute to ECM-synthesis and –degradation, providing a 3-dimensional support scaffold for myocytes and other cells of the heart. In addition, they also produce and secrete growth factors, cytokines, and other signalling molecules (such as IL-1β, IL-6, and tumour necrosis factor (TNF)-α; reviewed in [6, 86, 87]). Recent reports have shown that another facet of fibroblast paracrine signalling is based on microvesicle (exosome) secretion by fibroblasts and subsequent cardiomyocyte uptake of these vesicles. These exosomes were shown to contain large amounts of miRNAs, including fibroblast-derived miR-21*. Neonatal rat fibroblast-derived miR-21* has been shown to target transcripts important for myofibril assembly *in vitro*, thereby potentially contributing to cardiomyocyte hypertrophy [88]. Interestingly, interaction with target cells of exosomes released by different cells (including heart cell lines) may involve connexin 43 (Cx43) coupling [89], a theme that will be revisited in more detail for fibrobast-myocyte interactions in sections 3.2 and 4.7.

More immediate ways in which fibroblasts influence cardiac function include direct biophysical signalling.

3.1 Fibroblast-Myocyte Biophysical Crosstalk

Although fibroblasts are electrophysiologically quiescent and unable to actively generate action potentials (AP), they are capable of **electrotonic coupling** to one another and to neighbouring myocytes, possibly contributing to trans-scar electric signal transduction.

While fibroblasts are electrically non-excitable (i.e. lacking current systems that can generate an AP upstroke), it is important to recognize that they contain an array of ion channels, exchangers, and pumps. Examples include voltage-gated K^+ channels, inward rectifying K^+ channels, large-conductance Ca^{2+}-activated K^+ channels, chloride channels (including cell-volume activated channels), voltage-gated proton channels, sodium-calcium exchangers, sodium-potassium ATPases, and stretch-activated channels [90-92]. The latter include BK_{Ca}, $K_{ATP,}$ and cation-nonselective stretch-activated channels, as well as the more recently described transient potential receptor family of ion channels such as TRPM7 [93], TRPV4 [93], and TRPC6 [94] (reviewed in more detail elsewhere: [95, 96]).

For roughly half a century, the presence of electrotonic coupling between cardiac fibroblasts and myocytes and the ability of fibroblasts to synchronise distant myocytes solely *via* passive signal conductionhave been well-established *in vitro*. Long-distance low-loss electrotonic conduction via fibroblasts is made possible by their high membrane resistance, combined with a relatively low membrane capacitance [97]. If a fibroblast is electrically coupled to a cardiomyocyte, the myocyte can therefore “AP-clamp” the fibroblast. As a result, the non-excitable fibroblast will passively display a myocyte AP-like potential, albeit with a slowed **upstroke** and reduced amplitude, as illustrated in **double whole-cell patch clamp** experiments in neonatal rat cardiomyocyte and fibroblast cell cultures [98]. *In vitro*, the signal attenuation in fibroblasts is small enough to allow conduction of a supra-threshold electrical signal over distances of up to 300 μm [99]. This mechanism may underlie the previously mentioned clinical phenomena of trans-scar conduction: fibroblasts could electrically couple both with myocytes and among themselves to carry activation across gaps in myocyte continuity [5, 35, 50, 100, 101]. Thus far, electrical signal propagation throughout scar tissue *in situ* has been observed experimentally in a handful of studies (Box 2).

3.2 Modes of Contact

Intercellular sites of connexins (Cx, mostly Cx43) involving fibroblasts are much smaller than those between muscle cells in the heart [13, 102]. Fibroblast-myocyte Cx co-localization has been observed in intact sino-atrial node, atria, atrio-venricular node and ventricles [102], as well as in sheep ventricular infarct tissue [13] (Fig. 1D). In the sheep model of infarct, Cx45-expressing fibroblasts appear in the damaged tissue within a few hours after MI and reach their peak density after 1 week, whereas Cx43-expressing fibroblasts emerge later and their numbers continue to rise until at least 4 weeks after infarction. Similarly, an increase in Cx43 levels of cultured fibroblasts obtained from infarcted versus normal murine hearts has been reported *in vitro* [103], supporting an increase in functional coupling between fibroblasts and neonatal myocytes in the dish [104].

Direct evidence for heterocellular coupling in native tissue has been published so far for rabbit sino-atrial node, where *Lucifer yellow* dye transfer between myocytes and fibroblasts was reported [105], presumably *via* Cx40 at homotypic fibroblast connections and Cx45 at heterotypic fibroblast-myocyte contacts [106].

Another possible domain of fibroblast-myocyte coupling is the perinexus, a specialised microdomain of hemichannels surrounding the Cx-dominated gap junction. In cardiac myocytes, this region contains elevated levels of Cx43 and the sodium channel protein $Na_V1.5$. Combined with narrow inter-membrane volumes at these sites, this could create the potential for cell-to-cell transmission of electrical activation at the perinexus *via* an electric field-based mechanism (ephaptic coupling) [107, 108].

Furthermore, electrical signal transmission between cardiomyocytes and fibroblasts may occur *via* tunnelling nanotubes (Fig. 1E). These are membranous, actin-containing conduits, 50–200 nm wide, that can link various types of cells independently of Cx (although Cx may be present at contact points between nanotubes arising from the different cells) over distances up to 300 µm [109-112]. Preliminary evidence for the presence of nanotube coupling between cardiac fibroblasts and myocytes has been reported in neonatal rat cells *in vitro* [113] and in a rabbit MI model *in vivo* [114]. Tunnelling nanotubes have been found to allow bi-directional propagation of calcium (in human myeloid cells [115]) and electrical signals (in rat kidney cells [116]). Nanotube coupling may also serve as a conduit for exchange of cytosolic and membrane-bound molecules and organelles, including mitochondria, at least *in vitro* [113]. This observation may offer an alternative explanation (alongside cell-fusion) to “trans-differentiation”, in experimental studies reporting traits that are genetically targeted to one cell type appear in a different cell population. The functional relevance of tunnelling nanotubes for cardiac structural and functional integration and repair remains to be established.

In addition to their coupling with cardiomyocytes, fibroblasts have also been shown to intimately interact with other cell types within the scar, including endothelial cells (for review see [117]), possibly *via* the cell surface molecule N-cadherin. Interactions with other cell types (e.g. immune cells) are likely, too. Interaction of fibroblasts with adipocytes within the scar has been suggested to affect conduction velocity *via* electrotonic source-sink alterations in human MI studies [19], although no mechanism of electrotonic coupling between these cell types has been identified so far [18].

Thus, fibroblasts are perhaps the most underestimated cell population in the heart. Given their versatility, they are an attractive – and, compared to cardiomyocytes, potentially more realistic – target for therapeutic intervention. The aim of such interventions would be to modify structure and function of cardiac scars for patient benefit.

4. Making Better Scars – Potential for Targeted Interventions

Attempts to encourage reprogramming of fibroblasts into myocytes have proven to be a problematical issue, which – if ever resolved – raises further questions about functional integration of the newly created cardiomyocytes within the heart. Also scarless healing is not necessarily a blessing, but rather a potential curse. This points to the need for "encouraging" the heart to make a better scar.

4.1 What, When, Where and How?

Translational work involving scar-modifying treatments aims to develop therapeutic approaches and delivery modes suitable both for planned and emergency interventions that will steer scar properties towards combining mechanical strength with desired levels of electrical integration. For post-MI scars, this could involve upregulation of fibroblast-based electrotonic coupling, to make scars electro-physiologically transparent. In contrast, for scars generated by ablation (and surgery) reduced levels of electrical coupling could allow one to make then permanently insulating. Thus, opposite 'electrical aims' may be desirable for diffusing the threat of arrhythmia post-MI and for improving the success of ablation.

Furthermore, repair would ideally involve fibroblast recruitment, activation, and retention in the scar, whilst reducing fibroblast activity in remote, non-infarcted areas of the myocardium. Several therapies to date have aimed at (among other targets) influencing the fibrotic response to injury. The most widely-used targets include angiotensin-converting enzyme and AT1 receptors antagonists, beta blockers, endothelin antagonists, and statins (reviewed in [4]). Regulation of cardiac fibroblast activity is not, however, the primary target of these pharmacological agents, but an off-target benefit. Other, more recent attempts to influence fibroblast activation involve anti-IL1 approaches (in human post-MI remodelling [118]), blocking *frizzled* signalling to prevent expansion of the fibrotic area in rat post-MI model [119], and interfering with TGF-β or Smad3 signalling (for review see [120]).

The development of more sophisticated, targeted interventions should consider the following questions: What should be targeted: which cell and which process? Where, either within or outside the scar, should one aim? When to target? How to target? As of now, the answers to these questions are far from clear

Cardiac fibroblasts at the site of injury are recruited from several sources and at different time-points post-injury. They represent distinct cell populations that may differ in their responsiveness to interventions. In addition, scar geometry may matter, and alteration of fibroblast function at the site of injury may have differential effects if applied to the centre or the periphery of a forming scar. Timing of interventions is equally critical. Many mediators involved in fibroblast activation are heavily implicated in other cellular processes (including other facets of cardiac repair). For

example, blocking TGF-β during the early post-injury phase could accentuate adverse remodelling by preventing timely resolution of the initial inflammatory process. On the other hand, 'too late' inhibition could be ineffective if advanced fibrosis and formation of a mature scar are no longer reversible. Thus, the window of therapeutic opportunity is unknown, and potentially narrow – both spatially and temporally

4.2 Targeting Recruitment

Therapeutic manipulation of the mechanisms involved in fibroblast recruitment from different sources may hold potential for modulation of cardiac remodelling and scar properties after injury. During the inflammatory phase of post-injury healing, chemokines such as MCP-1 provide key signals for recruitment of both inflammatory cells and activated fibroblasts (for a review see [121]). Cardiac-specific overexpression of MCP-1 improves post-infarct cardiac function and remodelling, at least in part by increasing fibroblast accumulation [79]. Furthermore, MCP-1 deletion in a murine angiotensin II-induced cardiac fibrosis model demonstrated reduced uptake and differentiation of circulating $CD45^+$ fibroblast precursors with resultant loss of interstitial fibrosis [122]. Therefore, influencing the homing of fibroblast progenitor cells (**fibrocytes**) to the site of injury may offer an interesting approach to modifying scar formation and remodelling. One should keep in mind, however, that like most chemokines MCP-1 has far-reaching activities that are fundamental to the post-injury inflammatory process (for example, macrophage recruitment and activity), and altering their actions may have severe side-effects.

An enticing proposal set forth here would be to engineer extracardiac cell sources to deliver genetic payloads for therapeutic benefit directly to the injury sites (Box 3). The ability to perform this delivery *via* autologous patient-derived cells may present a safe, reliable and efficacious mode for generation of electrically and mechanically improved scar properties with positive consequences on cardiac function.

Additionally, targeting fibroblast clearance from the scar [25, 123] might also offer a novel therapeutic aim. Strategies aimed at reducing myofibroblast apoptosis have reported favourable effects on infarct scar healing. For example, inhibition of Fas/Fas ligand interaction in mice 3 days after MI reduced apoptosis of fibroblasts and macrophages, resulting in a thick, elastic and highly cellularised scar, and in lessening of cardiac dysfunction and heart failure progression [85].

4.3 Targeting miRNAs

Making use of miRNA signalling (reviewed in [25]) may show promise, too. For example, miR-125b affects EndMT in the heart and potentially drives fibroblast generation during fibrosis progression, as suggested by studies using murine

endothelial cell cultures [124]. Additional *in vivo* and *vitro* models identified mir-125b as a regulator of fibroblast activation [125]. Preclinical studies involving manipulation of miR-21 and miR-29 have shown beneficial effects on post-injury cardiac remodelling in rodents. In a murine model of angiotensin II-induced hypertension, a miR-29 mimetic attenuated the development of cardiac fibrosis [126, 127], while miR-21 inhibition increased survival after MI [127] and suppressed the development of interstitial fibrosis, lessening cardiac dysfunction in a murine model of pressure overload [128]. Furthermore, miR-145 has been associated with fibroblast activation immediately after infarction in mice, as well as with production of mature collagen *in vitro*, again providing a potential target for modulation of endogenous scar formation [129]. Lastly, miRNA-30 and miRNA-133 have also been shown to modulate the deposition of collagen fibres in rat neonatal cardiomyocyte and fibroblast cultures [130]. Therefore, using specific miRNA to deliver therapies directly to selected cell types could be a tempting option for future clinical interventions.

4.4 Targeting Periostin

Another promising target is the peptide periostin, identified as a critical regulator of fibrosis [131]. It has been shown to alter the deposition and attachment of collagen, collagen fibre diameter and crosslinking, as well as mechanical adhesion between myocytes and fibroblasts. Additionally, periostin signalling promotes fibroblast migration and cytoskeletal contraction, creating more aligned, sturdy, and less rupture-prone scars [132, 133]. Periostin signalling improves cardiac function post-infarct, but it also leads to an overall increase in the level of fibrosis in mice [133] and pigs [134], which illustrates the sensitivity needed for targeted interference with existing signalling pathways.

4.5 Targeting Caveolin

Caveolin-1 (Cav-1), a protein associated with plasma membrane invaginations known as caveolae (although it is also present in other cellular membranes), is important for signal transduction and mechanosensing, and may be a therapeutic target in fibrotic diseases.

Cav-1 is a master regulatory protein that binds to and inhibits the function, or promotes the turnover, of kinases in a variety of signalling cascades. These include MAP and Src kinases, protein kinase C, G proteins, growth factor receptors, and Akt and TGFβ signalling [135-137]. Cav-1 is under-expressed in fibroblasts during the development and progression of fibrotic conditions in humans [138-141], and heart (and lung) fibrosis are observed in global Cav-1-deficient mice [142-144]. Cav-1 deficiency leads to overexpression of collagen due in part to the engraftment and hypermigration of circulating CD45+ monocytic cells in injured heart (and lung), due to elevated expression of chemokine receptors, and to an enhanced differentiation

of cells into activated fibroblasts [141]. Cav-1 appears to be an amenable target for corrective intervention, as viruses encoding full-length *cav-1*, or a Cav-1 scaffolding domain peptide (amino acids 82-101 of cav-1) [145, 146] can prevent fibroblast activation.

4.6 Targeting Scar Mechanics

The myocardial collagen network can be modified to adapt to mechanical conditions. Interestingly, collagen production and deposition alone may not be sufficient, as it is collagen cross-linking that solidifies the scar and gives it its resilience and stability [20, 147, 148]. Concurrent with cell proliferation, activated scar fibroblasts produce lysyl oxidase (LOX) enzymes, which strengthen and stiffen the collagen network by crosslinking fibres [149]. Inhibition of LOX modulates collagen accumulation and maturation in a model of murine infarct and improves cardiac function, identifying LOX family members as a plausible target for intervention [150, 151]. Additionally, targeting collagen fibre orientation can affect overall scar stiffness by making scars more (or less) isotropic [20, 152].

Another option for intervention is targeting **infarct expansion** – the combined thinning and dilatation of infarcted tissue. Expansion, apart from being detrimental to cardiac mechanical efficiency, is associated with increased risk of infarct rupture in human [153]. By developing the means of stimulating infarct compaction, one may be able to strengthen cardiac tissue and improve ventricular geometry. This putative effect could perhaps be achieved by increasing collagen cross-linking inside, and maintaining it unchanged outside, the scar zone.

In any case, and whatever targeting mechanism may eventually emerge as clinically promising, the scar's mechanical function must *at least* be preserved.

4.7 Targeting Myocyte-Fibroblast Coupling

The making of better scars may require targeted control of fibroblast-myocyte electrotonic coupling. Coupling between fibroblasts and cardiomyocytes can be arrhythmogenic in rodent *in vitro* cultures [154-157]. Computer modelling suggests that this could be a consequence of fibroblasts acting as **current sources/sinks** [158-160]. In contrast, fibroblasts, genetically engineered to overexpress Cx43 have been shown to have anti-arrhythmogenic effects on cultured cardiomyocytes, offering an electro-tonic buffer that supresses spurious excitation [161]. Injection of (natively Cx43-expressing) fibroblasts, overexpressing voltage-sensitive potassium channels (Kv1.3), into rat heart tissue reduced automaticity and prolonged refractoriness *in vivo* [162].

Research is also underway to design peptides that prevent closure of Cx43 gap junctions between myocytes [163]. Here, spatial and temporal control will again be

crucial, as Cx also contribute to the spread of acute injury signals [164]. This novel treatment could be extended to involve targeting hetero-cellular fibroblast-myocyte gap junctions.

In terms of improving scar properties *in vivo*, it would be desirable to prevent trans-scar conduction after atrial ablations (plausible target: down-regulation of heterotypic Cx), while post-MI it may be beneficial to increase it to make ventricular scars electrically transparent (plausible target: up-regulation of heterotypic Cx-coupling). This would involve enhancing the fibroblasts' ability to act as a passive conductor of supra-threshold stimuli between otherwise isolated cardiomyocytes, homogenizing activity and preventing the development of barriers that favour re-entry.

The huge potential benefit of this concept has been confirmed in whole animal experiments with transplantation of autologous Cx43-overexpressing myoblasts into infarcted rats, an intervention which decreased the occurrence of arrhythmias [165, 166]. The key question will be: how to deliver the required message (e.g. up- or down-regulation of heterotypic Cx) to the right site, at the right time? For a proposal – see Box 3.

Conclusion: to Better the Heart – Make Better Scars!

There is a call for a revised conceptual approach to cardiac electrophysiology. Fibroblasts should be considered as not only a "silent" population of cells generating biochemical factors and structural proteins, but rather as a heterotypic and dynamic community of active participants in shaping cardiac structure and function. Due to their abundance, strategic location, phenotypic plasticity, ability to communicate *via* various mechanisms with a wide range of cells, and active participation in cardiac mechanical and electrical activity, cardiac fibroblasts are well-suited as key effector cells for cardiac repair and regeneration. Understanding the phenotypic and functional characteristics of fibroblasts in relation to cardiac function is crucial for the design of therapeutic strategies to treat the injured heart. One can envision gene-targeting, (stem) cell transplantation and/or reprogramming, as well as novel pharmacological approaches to modulate post-injury remodelling. The potential to steer the naturally occurring reparative processes is also conceptually pleasing (and promising, see Outstanding Questions). In that sense, the fact that at least some stem cells therapies have yielded fibroblast- rather than cardiomyocyte-like cells in myocardial infarcts is not necessarily a defeat, but perhaps an electrifying start.

BOX 1. Not all scars are created equal.

In myocardial infarction, oxygen starvation preferentially eradicated the more metabolically-active muscle cells, so that locally surviving cells, with a bias towards non-myocytes, will contribute to scar formation.

Ablation, whether by radio-frequency (increased temperature) or cryo-interventions (decreased temperature) is non-selective in destroying cells, so the vast majority of cells forming the scar invade from intra- or extra-cardiac sources outside the ablated tissue volume, although some of the original extracellular matrix (ECM) will remain present.

Finally, post-surgery scars involve *de-novo* ECM generation and cellularisation.

Insight into differences in scar formation in these settings is currently limited.

BOX 2. Evidence for fibroblast-myocyte electrical coupling in cardiac scar tissue.

Optical mapping of voltage-sensitive dye signals in fully-transmural infarcts in left ventricles of adult rabbit hearts revealed evidence of cardiac excitation wave propagation into scar tissue, even after chemical ablation of any surviving sub-endocardial muscle layers [167]. The signals from within the scar resembled ventricular AP, albeit with slowed upstroke and reduced amplitude – as seen in cell pairs [98], and subsequently reconfirmed in work by other labs [168, 169]. These AP-waves were not accompanied by changes in intracellular free calcium concentration [168] - a signature activity of cardiomyocytes. Therefore, the most likely scenario would involve non-myocytes conducting the electrical signals within the scar.

However, since the scar contains surviving myocytes, which could (at least in theory) form a convoluted set of continuous pathways, studies using dyes that stain cells indiscriminately of their type are not strictly conclusive [170].

First *conclusive* proof of fibroblast involvement in electrical AP-transmission in scar tissue comes from the use of genetically-encoded voltage-sensitive fluorescent protein 2.3 (VSFP2.3), expressed in murine heart to monitor transmembrane potential in fibroblasts only. In the border zone of fully healed post-**cryoinjury scars**, cardiomyocyte-like AP waveforms were reported by VSFP2.3, even though the reporter protein was expressed *solely* by fibroblasts [171]. This confirms the possibility of AP transfer from cardiomyocytes to non-myocytes in post-injury native heart issue.

BOX 3. Delivering therapeutic payloads straight to the heart of the injury.

A potentially interesting way of delivering relevant payloads to the forming scar is to use BM transfection (virus injection into the BM), which – if timed appropriately – could cater for the required targeted delivery of therapeutic interventions, at least to a significant proportion of fibroblasts involved in the post-injury response.

This builds on the observation that BM-derived fibroblasts make major contributions to post-injury scar formation (see section 2.3) [71]. This was irrefutably proven using chimeric mice, where the BM of irradiated animals was reconstituted by a *single clone* of GFP+ hematopoietic stem cells. All BM-derived cells could thus be tracked with certainty, and they were found to give rise to bona-fide GFP-positive fibroblasts/myofibroblasts in cardiac scars [72]. The therapeutic potential of the approach was shown using lentiviral vectors to silence periostin (which promotes fibrogenesis), injected into the BM after ventricular (cryo-)injury to mimic acute emergency settings, reduced scar size and fibrosis, and stabilised performance metrics (e.g. ejection fraction) to values comparable to normal baseline [55].

Therapeutic vectors will not only have to drive sufficient expression of relevant gene products, but be 'self-terminating', and specific for connective tissue, ideally with prevalence for the heart, as long-term and/or effects on other organ systems need to be benign or absent.

One way of achieving this for planned procedures, such as catheter-based ablation, would be to prime the body *via* short-lived BM-transfection with protein expression constructs that are sensitive to the biophysical environment. These could then be activated intra-procedurally at the site of ablation, for example by heat (temperature-sensitive expression trigger) or light (optogenetically encoded message).

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Glossary

atrial ablation lines; lesions introduced by local energy delivery, usually *via* intracardiac catheters, aimed at interrupting re-entrant atrial excitation wavelets, such as in atrial fibrillation

connexins; transmembrane proteins that assemble in groups of six to form a connexon hemichannel; two hemichannels from adjacent cells can form gap junctional channels connecting the two cytosols

cryoinjury; a procedure to induce cardiac injury, using (usually liquid nitrogen-) cooled probes of consistent size and shape

current source/sink; descriptive term that refers to an electrically connected membrane system that may accelerate (source) or slow (sink) electrophysiological changes in a cell

double whole-cell patch clamp; electrophysiological method simultaneously using two patch clamp electrodes to characterize junctional membrane conductances in cell pairs

ejection fraction; the fraction of total chamber volume (occasionally given as a percentage instead) that is pumped out during contraction

electrotonic coupling; direct spread of current between neighbouring cells (without a pore-requirement for generation of new action potentials)

fibrocyte; transitional cells that express leukocyte markers such as CD45 (indicating bone marrow origin) as well as mesenchymal cell markers (such as collagen I)

fibrosis; is the formation of excess fibrous connective tissue in an organ or tissue, such as during reparative or reactive processes

infarct expansion; acute regional dilatation and thinning of the infarct zone

optical mapping; fluorimetric method of measurement of activity-reporting signals (for example using voltage-sensitive fluorescent dyes) in cells or tissue

pressure overload; pathological state in which the heart has to contract while experiencing an excessive afterload

re-entry of excitation; an situation when a propagating wave of electrical excitation fails to die out after normal activation and persists to re-excite the heart in an irregular manner

transmural scars; injury-induced tissue remodelling involving scar formation through the entire thickness of the cardiac wall

upstroke; depolarisation phase of the action potential

volume overload; pathological state in which the heart has to contract while experiencing an excessive preload

Figure 1. Cardiac scars are very much "alive". Representative microscopy images of fibrotic cardiac tissue in humans, sheep and mice. **(A)** Different types of human cardiac fibrosis in explanted hearts, with varying landscapes of collagen-dense areas (red – collagen stained with picrosirius red, visualised by light microscopy). Interstitial fibrosis is an accumulation of collagen between groups of cardiomyocytes; in diffuse fibrosis short collagen septa are interspersed among myocardial fibres; patchy fibrosis involves lateral separation of cardiomyocytes over relatively long distances; compact fibrosis is characterised by large dense areas of collagen that are completely devoid of cardiomyocytes. Note: assessment of cardiac scarring using collagen staining creates an illusion of the scar being "acellular" (especially in the case of compact scars, such as seen post-myocardial infarction). From [156] with permission; scale bar = 1 mm. **(B,C)** Healed post-MI scars contain large numbers of non-myocytes, intermingled within collagen fibres. B: non-myocytes (N-M; including fibroblasts, endothelial cells, lymphoid cells) labelled with anti-

vimentin antibody in infarct zone of a 30d-old sheep infarct, visualised by confocal microscopy. From [13] with permission; scale bar = 40 µm. C: electron micrograph of a murine infarct zone, showing thick collagen bundles interspersed with non-myocytes. Scale bar = 10 µm. **(D,E)** Fibroblasts may form different forms of electrically conducting connections with myocytes. D: 30d-old sheep infarct border zone labelled with myomesin (staining cardiomyocytes, red), vimentin (non-myocytes, F, blue) and Cx43 (green), visualised by confocal microscopy. Non-myocytes express Cx43 at point of contact with myocytes (arrowheads). From [13], with permission; scale bar = 40 µm. E: electron micrograph showing tunnelling nanotubes between non-myocytes (N-M) and myocytes (M) at the murine post-cryoablation scar border, visualised by electron microscopic tomography. Scale bar = 1 µm.

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- What are the origin and sub-types of cardiac fibroblasts?
- How can we identify and trace them during normal development, homeostasis, disease, injury, and repair?
- Rather than using exogenous interventions, can we build on natural post-injury repair mechanisms, present within the heart, to improve repair?
- Is it possible to steer cardiac self-repair to provide mechanical strength and prevent electrical malfunction in post-injury tissue?
- What are the modes of fibroblast-myocyte biophysical coupling, when and where do they occur, how are they regulated, and in what setting do they matter?
- How can we harness new emerging technologies (i.e. novel therapeutic approaches including gene targeting or the use of photo-activated proteins) to engineer better scars?
- Can we use our current knowledge of scar mechanics and secretome information to contribute to the development of improved, potentially patient-specific biomaterials (patches, injectable polymers) for surgical heart repair?

Key Figure

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